

Humans: the ultimate animal models

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HUMANS ARE THE ULTIMATE ANIMAL MODELS OF HUMAN DISEASES, BUT AS HUMANS THERE ARE LIMITATIONS IN THEIR USE, PARTICULARLY TO INVESTIGATE INHERITED DISEASES AND TO DEVELOP THERAPIES. WE NEED TO EXPLORE HOW WE CAN OPTIMISE THE USE OF BOTH HUMAN AND NON-HUMAN MODELS IN UNDERSTANDING INHERITED DISEASES AND DEVELOPING THERAPIES, BUT ALSO TO BE INNOVATIVE IN DEVELOPING NOVEL WAYS OF STUDYING HUMANS.

As clinicians, our clinical practice revolves around our interactions with patients. We obtain a history, perform an examination, investigate appropriately, make diagnoses, and instigate and monitor a treatment plan. It may therefore seem obvious that humans should be the ultimate animal models to use to further our understanding of the causes and treatments of human diseases. The COVID-19 pandemic brought this sharply into focus. When confronted with a major new pandemic in humans, urgent clinical studies, epidemiological studies and therapeutic trials in humans were necessary alongside the crucial laboratory studies to bring the pandemic under control. Luckily pandemics on this scale are extremely rare compared with many of the diseases we deal with, including most inherited neurological diseases which are often chronic and disabling. The limitations in using humans as disease models, especially in therapy development, has necessitated the development of multiple other *in vitro* (immortalised cell lines and human induced pluripotent stem (IPS) cells) and *in vivo* (including invertebrate (*Caenorhabditis elegans* (roundworm), *Drosophila*) and vertebrate (zebra fish, rodent and non-human primate) disease models. While these have been and remain invaluable, there are limitations to all these preclinical models as shown by the number of therapies developed and successfully tested in animal models that then fail in human clinical trials.¹

The last 25 years has seen an explosion in the understanding of the genetic basis of diseases and especially neurological diseases. The increasing identification of new genes has been accelerated by the development of next generation sequencing techniques, especially whole exome (WES) and whole genome sequencing (WGS). In the area of inherited neuropathies there are now over 100 causative genes.² In one of these diseases, TTR amyloidosis, gene silencing therapy is now in clinical use and in many others clinical trials of a range of therapies are ongoing.^{3,4} Careful phenotyping of patients has underpinned all these genetic discoveries. In the inherited neuropathies as in many other genetic diseases, animal models have been critical in the study of disease pathogenesis and efficacy of

novel therapies, but it is increasingly clear that these animal models are not “mini humans” and usually are best thought of as models of pathways rather than the complete human disease.

It is timely to review how we approach the study of inherited diseases and to explore how we can optimise the use of human models in understanding the pathogenesis and in the development of therapies for human diseases.

INHERITED NEUROPATHIES

The inherited neuropathies are an ideal paradigm for studying inherited diseases as they are common, affecting between 1:2500 and 1:10 000, and encompass the complexity of genetic diseases both clinically from pure neuropathies to complex neuropathies and genetically including autosomal dominant (AD), autosomal recessive (AR) x-linked and mitochondrial inheritance.^{5,6} Like many inherited diseases they are caused by a range of mutations from single gene disorders (eg, Charcot-Marie-Tooth disease (CMT1A) due to peripheral myelin protein 22 variants (*PMP22*)) to complex large repeat intronic expansions (eg, *CANVAS* (cerebellar ataxia, neuropathy, vestibular areflexia syndrome) due to an expansion in *RFC1*).²

There is no perfect way to classify the inherited neuropathies but a simple classification into sole neuropathies, where the neuropathy is the sole or predominant disease manifestation (eg, Charcot-Marie-Tooth disease (CMT)), and complex neuropathies, where the neuropathy is part of a more generalised neurological or multisystem disorder (eg, the mitochondrial diseases), is useful. However, the use of next generation sequencing has identified a number of complex neuropathies where the patient can initially present with a CMT-like neuropathy, further emphasising the need for an accurate genetic diagnosis in all patients (figure 1).⁷

In recent years the term CMT is increasingly used to include classical CMT with motor and sensory involvement but also the related disorders, hereditary motor neuropathy (HMN), a pure motor or motor predominant form, and hereditary sensory neuropathy (HSN), a pure sensory or sensory predominant form (figure 1).² Classical CMT is divided into the demyelinating type (CMT1) with upper limb motor conduction velocities below 38 m/s and the axonal form (CMT2) with velocities above 38 m/s, with intermediate CMT being used to define patients with intermediate conduction velocities usually between 25–45 m/s. Although there have been more than 100 genes described to cause the inherited neuropathies,⁷ the most common type of CMT accounting for over 50% of all cases in most populations, including the UK,



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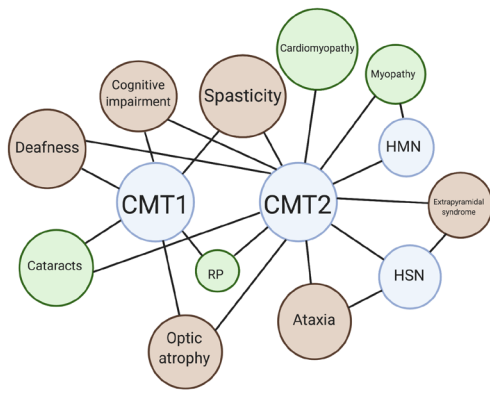


Figure 1 Phenotypes associated with inherited peripheral neuropathies. Charcot-Marie-Tooth (CMT) disease types 1 and 2 are defined by a predominant demyelinating or axonal peripheral neuropathy, respectively. Hereditary motor neuropathy (HMN) and hereditary sensory neuropathy (HSN) represent phenotypic extremes and show significant overlap with CMT2. Many other more complex neurological diseases such as hereditary spastic paraplegia, ataxia and optic atrophy may also develop a peripheral neuropathy and in a minority this may be the presenting feature. RP, retinitis pigmentosa.

USA and northern Europe, is CMT1A due to a 1.4 Mb duplication of chromosome 17 containing the *PMP22* gene.⁸ The frequency of subtypes varies in different populations especially in populations with a high rate of consanguinity where AR forms are more common.

Humans

Humans clearly differ from other species and it is beyond the scope of this commentary to give a detailed description of the evolution of humans and how they differ. Humans are members of the Hominidae family and are most closely related to the pan genus (Chimpanzees), but for understandable reasons primates are rarely used as animal models. Rodents, including mice and rats, are the most common mammalian species used to study human diseases and the differences between humans and rodents pose problems in modelling diseases. Using the inherited neuropathies as an example, the challenges in modelling human neuropathies in rodents include:

- ▶ Most inherited neuropathies are length dependent and humans are tall—a 1.8 m man will have lower limb nerves of up to 1 m in length. The inability to model neuropathies in nerves of this length is a major limitation in understanding the pathogenesis and developing therapies.



Figure 2 A disease model must mirror the changes that occur in the human body during normal ageing.

- ▶ Humans have a long lifespan compared with most other species, especially rodents that are frequently used to model inherited neuropathies. This poses particular challenges in modelling inherited diseases. We are only at the early stages of understanding the complexity of the genetic determinants of development at each stage of the human cycle (figure 2). The genes important for fetal development are likely and in some cases shown to be different from those needed for early childhood development. The growth spurt seen during puberty and adolescence will involve genes particularly important for neuropathies as they need to allow for the growth of long axons. In adulthood and older age the genes needed to maintain and repair cells will be increasingly important. Which genes are important at each stage of the human cycle and whether key genes change their function at different stages of the cycle is important to understand as this will influence the phenotypes seen with different mutations. We may not only need to model genetic diseases but we may need to model diseases at time specific points in the human cycle to get a true picture of a disease.
- ▶ As with all species, peripheral nerves do not exist in isolation and in humans the environment they exist in will be unique. As an example, during puberty where a lower limb motor nerve may need to grow up to 1 m in length, this nerve will grow alongside many other tissues including blood vessels, tendons, ligaments, muscle and skin. Many useful observations about neuropathies have been made from studying axonal degeneration and regeneration after nerve injury using a variety of models, but it is impossible to model the exact environment of human nerve growth. The recent development of induced pluripotent stem cells (iPSC) has been a major advance in allowing post-mitotic human cells to be used to study diseases, but to date these cells when differentiated into neurons are unable to reliably model aged nerves and are also unable to model the complex multicellular in vivo environment.⁹
- ▶ The diversity of phenotypes seen with inherited neuropathies include classical length dependent neuropathies as seen in CMT1A, neuropathies with upper limb predominance (*GARS*, *BSCL2*), neuropathies with a high incidence of diaphragmatic involvement (*GDAP1*) and vocal cord involvement (*GDAP1*, *TRPV4*), and neuropathies with diverse phenotypes such as congenital insensitivity to pain (*NTRK1*, *NGF*).² Rodent models of these mutant genes can be generated to have a neuropathy but it is very difficult to model the detailed phenotypes seen with these genes.

Gene discovery

Identifying the genetic basis of the inherited neuropathies as with other inherited diseases has been completely dependent on human studies and these discoveries have further advanced our understanding of the function of multiple proteins in health and in disease.

The first causative genes for CMT, including *PMP22*, myelin protein zero (*MPZ*), gap junction β (*GJB1*) and mitofusin 2 (*MFN2*) were identified by classic linkage studies in large families.^{10–14} Careful phenotyping including clinical examination, neurophysiology and neuropathology were critical in these early family studies. These studies not only identified the causative genes but also in some cases identified proteins that had previously not been known to be important in peripheral nerves. Mutations in *GJB1*, which codes for the gap junction protein, connexin 32, cause x-linked CMT and this discovery led to the

identification of these important gap junctions that allow rapid transport of ions and small molecules in peripheral nerves.¹³

The next generation sequencing techniques, WES and WGS, revolutionised our ability to identify causative genes, allowing these discoveries to be made in smaller families which were often not suitable for classic linkage studies. Careful human phenotype observations were still critical as exemplified by the identification of bicaudal D homolog 2 (*BICD2*) as a cause of the rare inherited neuropathy, spinal muscular atrophy lower extremity predominant (SMALED).¹⁵ As is common in WES studies a large list of candidate genes were initially identified in the original family studied; however, it was the observation that the family had an identical phenotype, including a specific lower limb muscle MRI pattern, to those families with mutations in dynein (*DYNC1H1*),¹⁶ the major protein in the retrograde axonal transport complex, that enabled the identification of *BICD2*, a dynein adaptor protein, as the causative gene. Furthermore, the observation that this *BICD2* related lower limb predominant neuropathy was congenital and non-progressive led to the recent identification of a non-cell autonomous mechanism of motor neuron loss resulting from impaired secretion of muscle derived neurotrophins during development.¹⁷ This has important implications for future therapeutic approaches in SMALED.

WGS has allowed human studies to be taken a step further in gene discovery, especially in the identification of complex non-coding mutations. The use of multiple carefully examined unrelated families with the complex neuropathy, CANVAS, together with WGS, allowed the identification of a biallelic intronic AAGGG repeat expansion in the replication factor C subunit 1 (*RFC1*) gene as the causative mutation.^{18 19} Multiple further studies reveal this to be the most common cause of late onset ataxia with an allele frequency ranging from 1–5% in the healthy population.^{19 20} This repeat expansion—like others such as the biallelic expansion of GAA repeats in intron 1 of the frataxin (*FXN*) gene which causes Friedreich's ataxia and many of the dominant spinocerebellar ataxias related to other repeat expansions—causes a complex neurological phenotype often with a predilection for large fibre sensory neurones, the cerebellum and pyramidal tracts. These clinical observations in humans raise the intriguing question as to whether the phenotype is linked in some way to the type of mutation as well as the host protein, as has been shown to be the case in myotonic dystrophy 1 (DM1) where the causative expanded CUG repeat RNA in the dystrophin myotonia protein kinase (DMPK) RNA sequesters multiple

RNA processing proteins leading to a multisystem disorder, rather than due to loss of DMPK function itself.²¹

One of the major challenges in genetics today is validating new mutations and new genes. New genes are particularly important as once a gene is reported in the literature (regardless of how carefully the authors report the finding) as a potential cause of a disease it is difficult to remove it. Many novel genes are reported in small individual families. These reports remain very useful as long as the genes reported are not claimed to be causative but to be candidates awaiting further validation. Next generation sequencing has allowed large human databases to be generated which are proving very useful in this regard, especially in helping show that some reported candidate genes are less likely to be disease causing (table 1). Our group reported a family with a mutation in methionyl-tRNA synthetase (*MARS*) as a potential cause of autosomal dominant CMT2, but stressed we had only found this gene in one family without full segregation.²² Since then, despite other individual small families being reported, human databases have suggested this is less likely to be a causative gene for CMT2 as it has now been shown that the original mutation is more prevalent in healthy population databases than the prevalence of the disease in the population.²³

THERAPIES FOR THE INHERITED NEUROPATHIES

Therapies for inherited diseases can be broadly divided into three approaches:

1. Genetic therapies

These include the gene silencing and gene correction therapies with antisense oligonucleotides (ASO), silencing RNA (siRNA) and CRISPR-cas9, and gene replacement therapies including viral vector gene replacement. These genetic approaches are attractive as they are disease agnostic and can be and are being successfully developed for a range of diseases including the complex inherited neuropathy, TTR amyloidosis (ASO, siRNA in humans) and CMT1A (ASO in rodent models).^{3 4 24}

2. Pathogenesis derived therapies

This is the classical pathway to therapy development where the exact pathogenetic mechanism of a disease is worked out and a therapy developed specifically to address this mechanism—for example, enzyme replacement therapies. Although there are no pathogenetic derived therapies in routine clinical use for the inherited neuropathies to date, the pilot studies in mice and humans using serine to reduce the

Table 1 Summary of genes reported to cause Charcot-Marie-Tooth disease but for which the frequency of the mutation in the population database, gnomAD, is more common than the frequency of the disease in the general population

Gene	Phenotype	Mutation	Mode of inheritance	Observed AC in gnomAD	Observed LOF AC* in gnomAD	Predicted maximum AC in gnomAD
Missense mutations						
<i>NAGLU</i>	CMT2	p.I403T	Dominant	3	–	1
<i>MARS</i>	CMT2	p.R618C	Dominant	3	–	1
		p.P800T	Dominant	9	–	1
<i>HSPB3</i>	HMN	p.R75	Dominant	177	–	1
Haplo-insufficiency						
<i>DHTKD1</i>	CMT2	p.Y485*	Dominant	1	145	1

For example, gnomAD contains information on approximately 250 000 alleles. If the population prevalence of CMT is at most 1 in 2500 of which 2% are due to rare types of CMT2 and of which 50% are due to a single mutation, one would expect 1 out of 250 000 alleles to contain the mutation of interest. The missense allele counts for *NAGLU*, *MARS* and *HSPB3* are in excess of this and argue that they are likely to be benign. Where haplo-insufficiency is proposed as the disease mechanism, one can expect that the total number of loss of function (LOF) alleles will be less than the prevalence of the gene in the population. For *DHTKD1*, the total number of LOF alleles in gnomAD is well in excess of the prevalence of the disease in the population.

AC, allele count; CMT2, Charcot-Marie-Tooth disease type 2; HMN, hereditary motor neuropathy; LOF, loss of function.

neurotoxic deoxysphingolipids (DSBs) in HSN1 secondary to *SPTLC1/2* mutations is an example of such a treatment approach.^{25–27} As with serine, drugs can be repurposed to be trialled as therapies.

3. Pathway therapies

As there are over 100 causative genes for the inherited neuropathies, with many genes only affecting a small number of families, development of treatments for pathways where many of the causative genes play a role such as axonal transport, protein folding and mitochondrial function is attractive. An example of this approach is the ongoing preclinical studies of histone deacetylase 6 (HDAC6) inhibitors to modulate axonal transport and other axonal functions in CMT2 and other axonal neuropathies.²⁸

Therapy development

Developing therapies for genetic diseases is complex and involves both the therapy development and the preparation of patients to be ready for a clinical trial—that is, trial readiness.

Trial readiness is obviously based on human studies including natural history studies and outcome measure and biomarker development; as with many other genetic diseases there has been major progress recently in this area in the inherited neuropathy field.²⁹

For all of the therapy development approaches described above, disease models are needed for multiple steps including understanding the pathogenesis of the disease, identifying candidate therapies and most importantly performing preclinical therapy trials. Although the development of IPS cells has been a major advance, we currently need non-human models for all of these steps; however, we need to realise the limitations of these models and to optimise their use to avoid the current poor rate of translation of preclinical trials of therapies for inherited diseases into successful therapies for patients.

Considerations include:

- ▶ Disease models, especially rodent models, are not “mini humans”. Regardless of how accurately a rodent model recapitulates a human disease, the complexity of humans, especially the length of axons in inherited neuropathies, cannot be exactly modelled. Despite these limitations, rodent and other models are crucial especially in early therapy trials and have allowed the use of larger animal models to be limited to critical studies. But the lack of successful translation of multiple therapies from the animal models to human trials suggest we need to be as rigorous in animal trials as we are with human trials. Although many authors have written about the need for appropriate disease models, adequate powering of studies, rigorous outcome measures and the need for study replication in independent laboratories, we still see too many clinical trials taking place in humans without adequate preclinical studies.
- ▶ To develop the best therapies we need to design better ways of doing preclinical studies in humans. Clearly we cannot do the kind of studies in humans that we can do in animal models, but the development of IPS cells has shown that we can develop models using human tissue that more accurately model human diseases. IPS technology is in the very early stages and it may well be that this can be developed to allow development of more accurate aged neurones and organoids. These models would be an ideal stepping stone for testing candidate therapies before moving to large scale clinical trials.

- ▶ We also need to continually improve our methods of studying human phenotypes, especially in rare diseases like some of the rare inherited neuropathies where large scale clinical trials are not possible. More detailed phenotyping may help gene discovery and therapy development—for example, studying disease extreme phenotypes can help identify disease modifying genes and open new therapeutic angles. Better phenotyping would improve the understanding of disease pathogenesis and progression in humans and inform therapy development and trial design decisions, especially trials in minimal numbers of patients. In the field of inherited neuropathies there have been phenotyping developments including nerve excitability studies,³⁰ MRI muscle and nerve,^{31 32} and skin biopsies to study dermal and epidermal nerve fibres, but much more focus in this area is needed.³³ We need to strive towards being able to phenotype humans in vivo at the molecular level. Ultimately we should aim at developing a live imaging molecular interactome to study pathways in humans throughout their lifespan, and although this is beyond today’s technology, the evolving developments in molecular imaging techniques suggest it will be possible one day.
- ▶ Optimising trial design for rare diseases is also crucial not only to get an accurate answer if a therapy works but to use the rare patient population optimally so as not to exhaust patient numbers in multiple parallel trials. We can learn from the adaptive design studies being done in many cancers such as prostate cancer.³⁴

CONCLUSION

Humans are the ultimate animal models of human diseases, but clearly there are major current limitations to how they can be used to study inherited diseases and develop therapies. Many other disease models are and will continue to be needed. There is a need to recognise the limitations of all non-human models and to continually optimise their use, especially in preclinical trials. Parallel to this is the need to develop better tools to study humans and to work with funders to recognise the need to continue to fund patient-based phenotyping and natural history research. With the inherited neuropathies as with many inherited diseases we are at a unique crossroad where we can begin a new era of studying the “natural history” of treated diseases often where these diseases were fatal—for example, spinal muscular atrophy type 1 and TTR amyloidosis. We must not lose this opportunity to continually learn from our patients and to enthuse our trainees with the excitement and value of patient-based research.

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